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Analysis of the disaccharides derived from hyaluronic acid and chondroitin sulfate by capillary electrophoresis with sample stacking

CE conditions for monitoring the unsaturated disaccharides of hyaluronic acid (di-HA) and chondroitin sulfate (di-CS) using an alkaline tetraborate buffer, electrokinetic sample injection, and UV absorption detection at 232 nm are reported. Separations were performed in an uncoated fused-silica capillary having reversed polarity and reversed electroosmosis generated with the addition of CTAB to the buffer. The influence of various separation parameters, including the concentration of CTAB, buffer pH, concentration of tetraborate, and applied voltage, on the resolution of the two disaccharides was investigated. Baseline separation was obtained with 25 mM tetraborate at pH 10.0 and having 0.05 mM CTAB. Chloride and phosphate in the sample are beneficial for the stacking of the disaccharides, with di-HA forming a much sharper peak than di-CS. Using samples prepared in 25 mM Tris-HCl (pH 7.5) and electrokinetic injection at the cathode at −10 kV for 40 s, linear relationships between the corrected peak area and the concentration of the disaccharides have been found in the ranges of 1.0–400.0 and 0.1–1.0 μg/mL (0.2–1.0 μg/mL for di-CS), with correlation coefficients being >0.9933 in all cases. The RSDs of detection times and corrected peak areas were between 1.13–1.24 and 1.57–2.13%, respectively. Applied to human serum samples that were prepared by ethanol precipitation and depolymerization of the two polysaccharides with chondroitinase ABC reveals comigration of endogenous compounds with di-HA and a sample-dependent detection time. The di-HA content in the serum sample can be estimated *via* subtraction of the blank peak that is obtained without enzymatic hydrolysis.

Key Words: Chondroitin sulfate; Electrokinetic injection; Hyaluronic acid; Serum; Stacking

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1 Introduction

Hyaluronic acid (HA) comprises negatively charged, linear polysaccharides composed of a variable number of repeating disaccharide units containing one hexosamine (β-(1→3)-*N*-acetylglycosamine) and one uronic acid (β-(1→4)-glucuronic acid). Together with heparin, heparin sulfate, dermatan sulfate, keratan sulfate, and chondroitin sulfate (CS), it forms the group of connective tissue polysaccharides known as glycosaminoglycans (GAGs). HA has a high molecular mass (up to 8×10^6 Da corresponding to about 10 000 disaccharide units), is not sulfated, and is not covalently bound to proteins [1–6]. HA is mainly produced by fibroblasts and other specialized connective tissue cells and participates in various cell-to-cell interactions. It is found extensively throughout the human body, being most abundant in loose connective tissues, such as the umbilical cord, synovial fluid, and vitreous body of the eye [7]. Synovial HA may pass into plasma *via* the lymphatic system [8]. In circulation, HA levels are maintained by an efficient receptor-dependent removal mechanism present in sinusoidal endothelial cells of the liver and by the enzymatic action of hyaluronidase [9]. Increased production of HA in the connective tissue surrounding the joints is stimulated by various inflammatory conditions and thus can be used to monitor joint condition [10, 11]. Increased serum HA levels may also arise from various liver diseases characterized by liver fibrosis and cirrhosis, due to decreased hepatic removal and/or increased hepatic production of HA during liver inflammation [12, 13].

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Abbreviations: CS, chondroitin sulfate; **di-CS**, α-4-deoxy-L-threo-hex-4-enopyranosyluronic acid-(1→3)-*N*-acetyl-D-galactosamine; **di-HA**, α-4-deoxy-L-threo-hex-4-enopyranosyluronic acid-(1→3)-*N*-acetyl-D-glucosamine; **EO**, electroosmosis; **GAGs**, glycosaminoglycans; **HA**, hyaluronic acid

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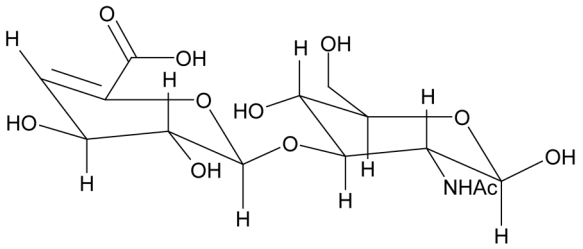
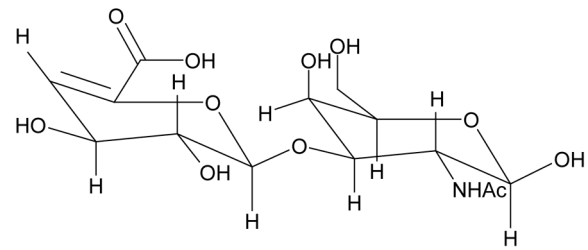
The determination of serum HA levels is useful for assessing the degree of liver fibrosis and for monitoring liver function [13–16] and thus is a topic of high interest for the treatment of patients in our departmental hepatology outpatient clinic. Monitoring serum HA is also used in other areas, such as in septic conditions, tumor growth (such as Wilm's tumor and malignant mesothelioma), and for the diagnosis of rare hereditary diseases (such as Werner's syndrome and cutaneous hyaluronanosis) [4]. Many different colorimetric, chromatographic, and electrophoretic methods have been developed for the analysis of HA and other GAGs, their metabolic products, and the degraded molecules thereof [1, 2]. Serum HA is currently determined with assays that are based upon the specific affinity of certain proteins to HA and that feature a low μg/L sensitivity [17], most notably a radiometric method (HA-test, Pharmacia, Uppsala, Sweden) and a sandwich enzyme-binding assay (Chugai Pharmaceutical, Tokyo, Japan; HA-test, Corgenix, Westminster, CO, USA). The radiometric assay measures the reactivity of the unbound reagent after removal from the sample using Sepharose-bound HA, while the enzyme-binding assay immobilizes HA to the surface of a microtiter well and uses an enzyme-tagged protein that is subsequently bound to the immobilized HA and catalyzes a color reaction that can be measured *via* absorbance at 450 nm. Both methods suffer from the tedious and laborious nature of the procedure, which prompted us to begin to search for new, more instrumental possibilities that are suitable to measure either entire or degraded HA molecules in human serum.

CE in its various modes is increasingly employed in the separation and analysis of a wide variety of carbohydrates, including HA and its degraded molecules [18–20]. CE with direct and indirect UV absorption detection has been applied to the analysis of intact HA in pharmaceutical semisolid formulations [21], in human and bovine vitreous humor [22], and in human synovial fluid [23]. CE has been used in determining the physical properties of intact HA by the addition of a polymeric matrix, such as pullan and polyethyleneglycol, to the separation electrolyte [24, 25]. HA of synovial fluid has been determined by CE after hydrolysis of the polymeric hyaluronan to the tetrasaccharide by the action of testicular hyaluronidase [23]. In other approaches, HA in effusions from human malignant mesothelioma [26] and in skin and other tissue samples of various species [27] has been degraded with chondroitinase ABC to the disaccharide α-4-deoxy-L-threo-hex-4-enopyranosyluronic acid-(1→3)-*N*-acetyl-D-glucosamine (di-HA, for chemical structure see Fig. 1) prior to analysis of di-HA by CE. Degradation of HA and CS with chondroitinase ABC is preferred as the resulting disaccharides, di-HA, and the nonsulfated disaccharide of CS, α-4-deoxy-L-threo-hex-4-enopyranosyluronic acid-(1→3)-*N*-acetyl-D-galactosamine (di-CS, for chemical structure refer to



disaccharide of hyaluronic acid (di-HA)



disaccharide of chondroitin sulfate (di-CS)

Figure 1. Chemical structures of the unsaturated disaccharide of HA (di-HA) and the unsaturated nonsulfated disaccharide of CS (di-CS) derived from HA and CS, respectively, through the action of chondroitinase ABC. NHAc refers to the *N*-acetyl group of the hexosamine moiety.

Fig. 1), contain unsaturated uronic acid residues at their nonreducing end allowing sensitive detection at 232 nm [1–3, 19, 26, 27]. This method has also been applied to the determination of HA of gastrointestinal carcinomas [28], and the CE analysis of GAG-derived disaccharides in biological samples has been reviewed by Lamari *et al.* [3]. HA has no unsaturated glucuronic acid and can thus only be detected at 190–200 nm, an approach that provides lower sensitivity and is prone to more interferences when analyzing biological samples.

Up to now, CE has never been used to analyze HA in human serum. The rather low HA concentration in serum (<100 ng/mL determined with the two routine assays in healthy subjects) requires efficient sample-stacking techniques for CE detection of intact or digested HA in serum. Human serum contains a high concentration of salts and this precludes online preconcentration techniques based on conventional sample-stacking or field-enhanced sample injection which require low salt matrices for efficient analyte focusing. Transient ITP or sample self-stacking [29–34], formation of a transient moving chemical reaction boundary [35], sweeping [36–38], and use of a dynamic pH junction [39], however, are online concentration techniques that can be used to temporarily induce narrow analyte bands in the presence of large amounts of salts.

In the present study, electrokinetic sample injection together with sample stacking was studied for high-sensitivity analysis of di-HA and di-CS in model mixtures and human serum digested by chondroitinase ABC using a borate buffer at alkaline pH, reversed electroosmosis (EO) provided by addition of CTAB, and direct UV detection at 232 nm. First, the effects of pH and concentration of the buffer, the CTAB concentration, and the separation voltage were examined. Then, the influence of injection time on resolution, peak area, peak height, corrected peak area, and theoretical plates was studied, and the impact of the chloride and phosphate concentration in the sample on sample stacking was investigated. The optimized method was applied to the determination of di-HA in sera of healthy humans.

2 Experimental

2.1 Reagents and solutions

All chemicals used were of analytical grade. HA (H-1751, as potassium salt, from human umbilical cord that contains small amounts of CS), di-HA (sodium salt), di-CS (sodium salt), and CTAB were purchased from Sigma (St. Louis, MO, USA). Boric acid, disodium tetraborate decahydrate, and ethanol (absolute) were from Merck (Darmstadt, Germany), and chondroitinase ABC from *Proteus vulgaris* (EC 4.2.2.4) was from Fluka (Buchs, Switzerland). A stock solution of the disaccharides of HA and CS was prepared in water (1.0 mg/mL each) and aliquots thereof were used to provide solutions containing 0.10, 0.20, 0.50, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, 100.0, 200.0, 400.0 μg/mL of di-HA and di-CS in 25 mM Tris-HCl (pH 7.5).

2.2 Instrumentation and running conditions

CE was performed on the Beckman P/ACE 5510 system (Beckman Instrument, Fullerton, CA, USA) that was equipped with a fused-silica capillary of 75 μm ID × 67 cm (60 cm to the detector; Polymicro Technologies, Phoenix, AZ, USA), which was mounted in a cartridge with a 100 × 800 μm aperture (Beckman). Prior to each injection, the capillary was successively rinsed with 1.0 mol/L NaOH for 2 min, distilled water for 2 min, and finally with running buffer for 3 min. If not stated otherwise, samples were injected electrokinetically at −10 kV for 40 s and separated at −12 kV. The temperature of the cooling fluid was set at 25°C. Detection by UV absorbance occurred at 232 nm. If not stated otherwise, the running buffer was composed of 25 mM disodium tetraborate that was titrated to pH 10.0 by addition of 1.0 M NaOH and contained 0.05 mM CTAB. All experiments were done in triplicate.

2.3 Serum sample treatment

The first part of the sample pretreatment is similar to that employed for the analysis of HA in effusions from human malignant mesothelioma [26]. Ten milliliters of absolute ethanol was added to 1 mL of serum and precipitation was completed overnight at 4°C. The precipitate was recovered by centrifugation using an Eppendorf microcentrifuge (15 000*g* for 5 min) and was dried at 40°C under a gentle stream of air. HA was digested *via* addition of 800 μL of chondroitinase ABC in 25 mM Tris-HCl (pH 7.5, 0.5 U/mL of enzyme solution) to the dried residue and incubated for 90 min at 37°C. Two milliliters of ethanol was added to the digested solution to precipitate the proteins and nondegraded polysaccharides. After centrifugation (3500*g* for 5 min), the supernatant was decanted into a clean glass tube, evaporated to dryness at 40°C under a gentle stream of air, reconstituted in 100 μL bidistilled water, and stored at −20°C. The preparation of the blank serum sample was identical except that the 800 μL of 25 mM Tris-HCl (pH 7.5) added for the incubation did not contain any enzyme.

3 Results and discussion

3.1 Separation of disaccharides of HA and CS

The CE determination of HA in serum as di-HA produced by depolymerization of HA with chondroitinase ABC requires the separation of the two similar nonsulfated disaccharides, di-HA and di-CS (as presented in Fig. 1), as well as sulfated disaccharides of CS. It is not difficult to separate the nonsulfated disaccharides from the disaccharides with monosulfate and disulfate since the charge-to-mass ratio is significantly different [26]. The electrophoretic separation of the two nonsulfated disaccharides (di-HA and di-CS), however, is a challenge as they have an identical net charge and the charge is located at the same position in each molecule. The only difference between these two disaccharides is the equatorial (di-HA) or axial (di-CS) position of the hydroxyl function on carbon 4 of the hexosamine residue (Fig. 1). Using the acidic phosphate buffer and reversed polarity as described in [26], the two disaccarides could not be detected in our laboratory. Thus, an alkaline borate buffer was used because of the complex formation between borates and carbohydrates and the additional advantage that the anionic borate disaccharide complexes formed have a higher UV absorbance than the free anions of the disaccharides [18, 40]. The impact of several experimental parameters, including the concentration of the cationic surfactant that is reversing EO, the pH and concentration of the running buffer, and the separation voltage, on the resolution of di-HA and di-CS was investigated using an aqueous sample containing about 2 μg/mL of each compound and electrokinetic sample injection. A typical electropherogram

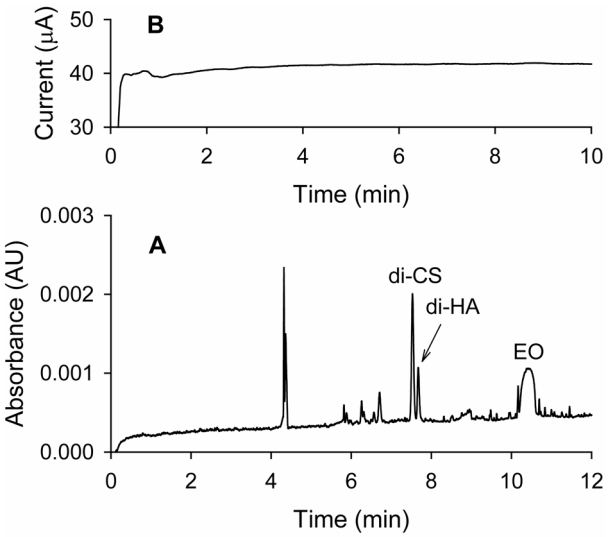


Figure 2. (A) Electropherogram and (B) temporal behavior of the current for the separation of di-CS and di-HA in presence of 0.05 mM CTAB. An aqueous sample with about 2 μg/mL of each disaccharide (di-HA from HA depolymerized with chondroitinase ABC) was analyzed. Conditions: capillary, 67 cm (60 cm to detector) × 75 μm ID; applied voltage, −15 kV; detection wavelength, 232 nm; electrolyte, 25 mM tetraborate, adjusted to pH 9.5 with NaOH; injection voltage and time, −10 kV for 20 s. EO refers to electroosmosis.

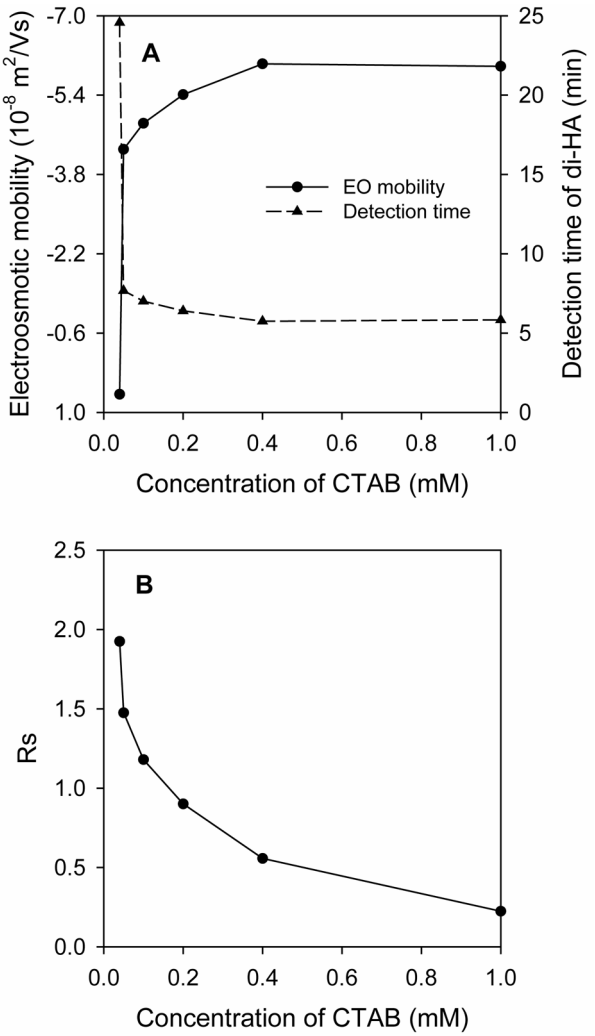


Figure 3. Influence of the CTAB concentration on (A) EO and detection time of di-HA, and (B) resolution of the disaccharides of HA and CS. EO refers to electroosmosis. Experimental conditions as for Fig. 2.

together with its current profile are presented in Fig. 2A and B, respectively. The sample solution contained 2 μg/mL of di-CS (standard compound) and about an equivalent amount of di-HA that was enzymatically derived from HA using chondroitinase ABC. As the HA standard also contained CS, the CS peak became somewhat increased. Furthermore, the peaks detected around 6 min could be those of sulfated disaccharides of CS [26].

There are two main reasons that are in favor of employing CTAB as buffer additive. Firstly, CTAB is an EOF modifier providing reversed EOF, which allows the use of electrokinetic sample injection of anions with a long injection time to improve the detection sensitivity. Secondly, *via* varying the CTAB concentration, the use of this cationic surfactant below its critical micellar concentration was found to improve the resolution of the negatively charged disaccharides of HA and CS. The data presented in Fig. 3 were obtained with CTAB concentrations between 0.04 and 1.0 mM and show the impact of the CTAB concentration on EO (Fig. 3A), the detection time of di-HA (Fig. 3A), and the resolution of the disaccharides of HA and CS (Fig. 3B) using 25 mM $Na_2B_4O_7$ (pH 9.5) as BGE. Resolution was noted to be highest at the lowest CTAB concentration. This can be explained with the increase of EO toward the anode as the CTAB concentration is increased. Accordingly, detection times and thus the time interval for separation decrease from about 25 to 5.9 min for 0.04 and 1 mM CTAB, respectively. Furthermore, the electrophoretic mobility of the two disaccharides might also be influenced by virtue of ion-pairing effects between CTAB and the negatively charged solutes as was previously investigated for di-CS and oligosaccharides derived from hyaluronan using tetrabutylammonium as ion-pairing agent [40]. Under the investigated conditions, CTAB concentrations between 0.04 and 0.05 mM were found to provide complete separation of the two disaccharides (Figs. 2A, 3B). At 0.04 mM of CTAB, the electroosmotic mobility was estimated to be $0.63 \times 10^{-8}\,m^2/V \times s$ (no reversal of EOF). The use of CTAB concentrations <0.04 mM resulted in electropherograms without the two disaccharide peaks being detected within a time interval of 30 min. Thus, 0.05 mM CTAB which provided an electroosmotic mobility of $-4.31 \times 10^{-8}\,m^2/V \times s$ (Fig. 3A) was selected for our project.

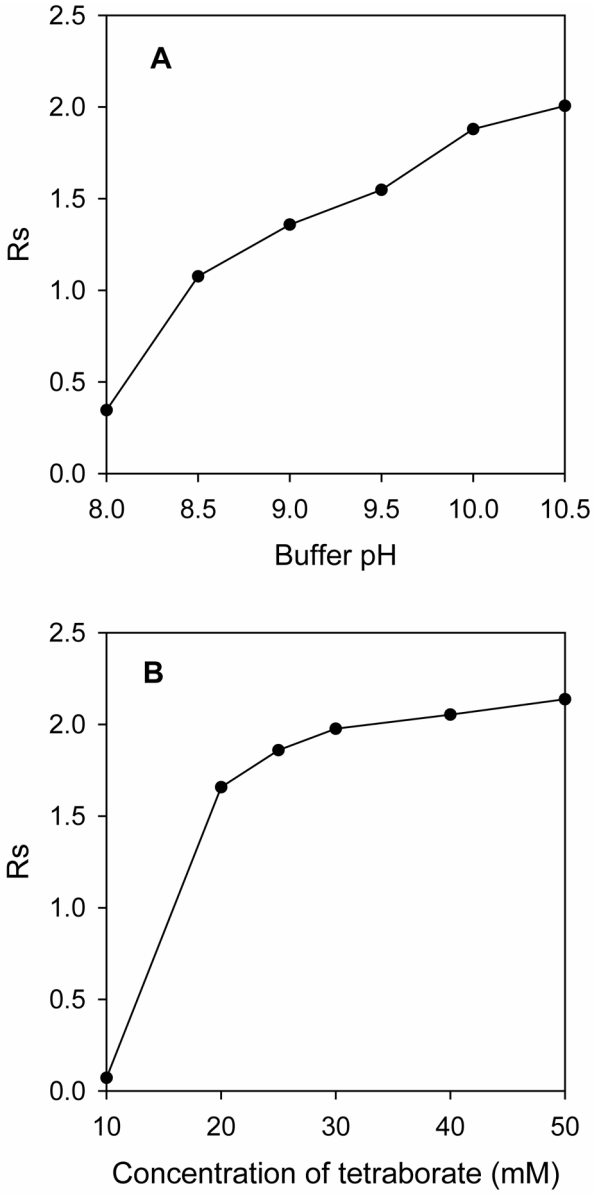


Figure 4. Influence of (A) pH and (B) tetraborate buffer concentration on resolution of the disaccharides of HA and CS. Buffers were composed of (A) 25 mM tetraborate containing 0.05 mM CTAB (pH adjustment with addition of boric acid or NaOH) and (B) tetraborate containing 0.05 mM CTAB and adjusted to pH 10.0 with addition of NaOH. Other conditions as in Fig. 2.

The impact of the buffer composition on resolution of the two disaccharides was studied *via* variation of buffer pH and concentration and having 0.05 mM CTAB as buffer additive (Fig. 4). The resolution was found to become larger upon increase of pH (Fig. 4A). Having a borate concentration of 25 mM, the two disaccharides are baseline separated for pH ≥ 9.5. A pH 10.0 buffer was further used because this pH offers a compromise between optimal resolution and buffer capacity. Both the resolution (Fig. 4B) and migration times of the analytes of interest increased upon increasing the buffer concentration. This is due to a decrease in EOF. Having a borate concentration above 20 mM provided a resolution >1.5. Furthermore, the current increased as the borate concentration was enhanced and reached about 129 μA for the 50 mM buffer. At currents above 85 μA (30 mM tetraborate), an instability of the system was noticed (slow increase of current with time). Thus, 25 mM was selected as buffer concentration for all further experiments.

With a 25 mM $Na_2B_4O_7$ (pH 10.0) BGE with 0.05 mM CTAB, the influence of the separation voltage on the resolution of the disaccharides of HA and CS, studied in the range between −10 and −18 kV, was found to be small. The resolution of the two disaccharides decreased with the increase of the separation voltage, namely, from 2.71 to 2.32 for −10 and −18 kV, respectively. The current increased from 46 to 92 μA. In order to avoid too high currents, a voltage of −12 kV was further used.

3.2 Sample stacking

To reach highest sensitivity, electrokinetic sample injection was used. The presence of chloride was found to nicely enhance the stacking of di-HA, an effect that was not noticed for di-CS (Fig. 5A). This difference in peak

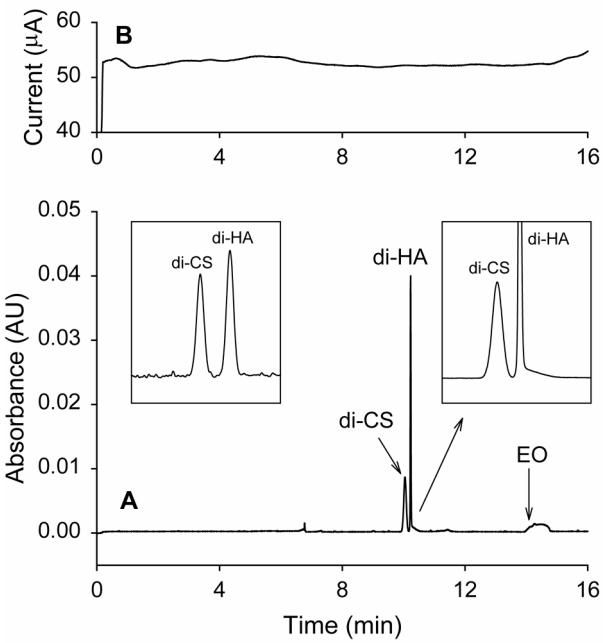


Figure 5. (A) Electropherogram and (B) temporal behavior of the current obtained for analysis of 20 μg/mL of each disaccharide in 25 mM Tris-HCl (pH 7.5) using the optimized experimental conditions given in Section 2.2. Inset to the right in panel A depicts a 1.2 min time interval of the two disaccharide peaks with expanded *x*-axis and *y*-axis scales. Inset to the left depicts data obtained with a sample containing the two disaccharides (2 μg/mL each) in water. EO refers to electroosmosis.

Table 1. Influence of the injection time of the sample on peak size, theoretical plates, and resolution[a)b)]

Injection time, s	Peak area		Peak height		*N*		R_s	Corr. area[c)]	
	di-CS	di-HA	di-CS	di-HA	di-CS	di-HA		di-CS	di-HA
10	1184	2554	296	656	121 237	168 172	2.418	102	214
20	2560	5060	639	1534	112 745	253 140	2.351	220	425
40	5528	10 324	1233	3550	94 207	372 240	2.012	483	883
60	8088	14 922	1530	5248	68 244	426 656	1.538	722	1309
80	11 420	22 408	1800	6082	47 380	423 880	1.044	988	1908

a) Experimental conditions as stated in Section 2.2 and Fig. 5A using a sample composed of 2 μg/mL of each disaccharide in 25 mM Tris-HCl, pH 7.5.
b) Mean values ($n = 3$).
c) Peak area/detection time.

shape is not observed when the two disaccharides are dissolved in water only (left inset in Fig. 5A; see also Fig. 2A). The two disaccharides were injected with time intervals from 5 to 80 s using a sample with 2 μg/mL of each disaccharide dissolved in 25 mM Tris-HCl (pH 7.5). The influence of sample injection time on peak area, peak height, the number of theoretical plates (*N*), the resolution between the two disaccharides, and the corrected peak area (peak area divided by detection time) is shown with the data presented in Table 1. As expected, an increase of the injection time led to higher peak areas, peak heights, and corrected peak areas for both compounds. The resolution between the two disaccharides and the peak efficiency of di-CS, however, became lower. Furthermore, contrary to the trend observed with di-CS, the peak efficiency of di-HA was found to increase as the injection time interval was enhanced up to 80 s. An injection greater than 60 s, however, was inappropriate because of the resolution. Therefore, 40 s was selected as the sample injection time (Fig. 5A). Using hydrodynamic sample injection, the detection sensitivity was found to be more than ten-fold worse (data not shown). Furthermore, field-amplified sample injection with application of a water plug prior to electroinjection from the sample dissolved in a low conductivity environment (water), as was previously documented to provide over 1000-fold sensitivity enhancement in other configurations [41], provided higher peaks for di-CS and di-HA (data not shown). This approach, however, was not effective with samples of high ionic strength, including the serum samples prepared in this work, and was thus not further investigated.

Upon analysis of depolymerized HA in samples prepared from human serum, the peak of di-HA was found to be extremely narrow (see below). This high efficiency was already noticed for the sample dissolved in Tris-HCl buffer (Table 1, Fig. 5A). In the absence of chloride, however, broader peaks were observed (left inset in Fig. 5A). Chloride and phosphate present in serum were previously identified as being beneficial for the stacking of analytes of like charge for which they act as transient stackers [33, 34]. These two anions were also found to enhance the stacking of the two disaccharides. Figure 6 shows data of peak width at half height ($W_{1/2}$) for samples of the two disaccharides in 25 mM Tris-HCl (pH 7.5) that were fortified with chloride (addition of NaCl, Fig. 6A) and phosphate (addition of KH_2PO_4, Fig. 6B). Without addition of NaCl, the chloride concentration in the sample was calculated to be 18.7 mM. Best stacking was obtained with addition of >10 mM chloride (total chloride concentration in the sample >28.5 mM) or ≥1 mM phosphate. Thus, the presence of chloride and phosphate in the serum (100 and 0.5–2 mM, respectively [34]) appears to be sufficient for the stacking of the disaccarides. More experimental work has to be conducted to identify the stacking mechanisms involved, particularly the more efficient stacking of di-HA compared to di-CS (Fig. 5A). In addition to sample self-stacking, it may very well be that sweeping with borate [37, 38] and/or pH induced stacking [35, 39] contribute to solute concentration as well.

3.3 Linearity, reproducibility, and detection limits

Under optimized conditions, the electroosmotic mobility was determined to be $-4.06 \times 10^{-8}\,m^2/V \times s$ (Fig. 5A). As the calibration range used was rather large, the linear relationship between the concentration of analytes and their corresponding corrected peak area was established in two ranges, namely, from 1.0 to 400 μg/mL and from 0.1 to 1.0 μg/mL (0.2–1.0 μg/mL for di-CS, see Table 2). The samples with the two disaccharides were prepared in 25 mM Tris-HCl at pH 7.5, *i.e.*, the buffer used for the chondroitinase digestion of HA in serum (Section 2.3), and analyzed under the optimized conditions (Fig. 5). Regression analysis yielded straight lines with $r > 0.993$ (Table 2). Reproducibility was determined by nine successive injections of a standard disaccharide solution within 1 day. For a sample with 10 μg/mL of each disaccharide, a good accuracy for the detection times and corrected peak areas was found with RSD being less than 1.3 and

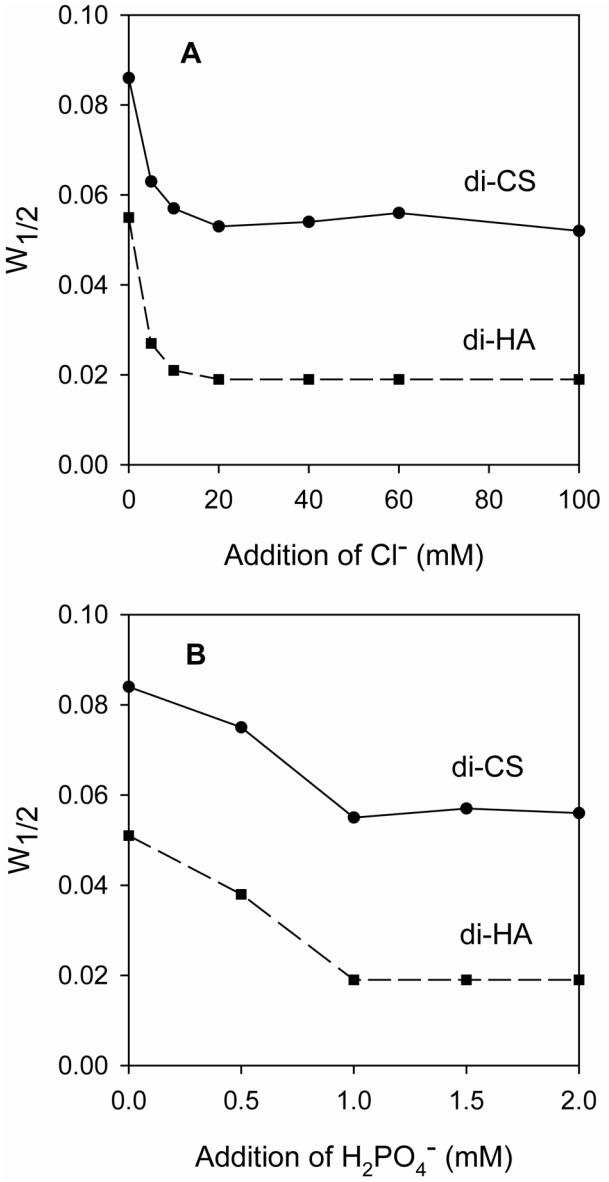


Figure 6. Influence of (A) chloride addition and (B) phosphate concentration in the sample on peak width at half height ($W_{1/2}$). Sample was composed of 10 μg/mL of the two disaccharides in 25 mM Tris-HCl (pH 7.5) and was fortified with NaCl or NaH_2PO_4 as indicated. Experimental conditions as for Fig. 5.

2.2%, respectively (Table 2). The detection limits (S/N = 3) of di-CS and di-HA were determined to be 0.10 and 0.075 μg/mL, respectively.

3.4 Analysis of di-HA in human serum after enzymatic depolymerization of HA

Chondroitinase ABC depolymerized HA in the serum of a healthy subject was analyzed with the optimized method and the obtained electropherogram is presented in Fig. 7B. The assigned peaks were identified by spiking the

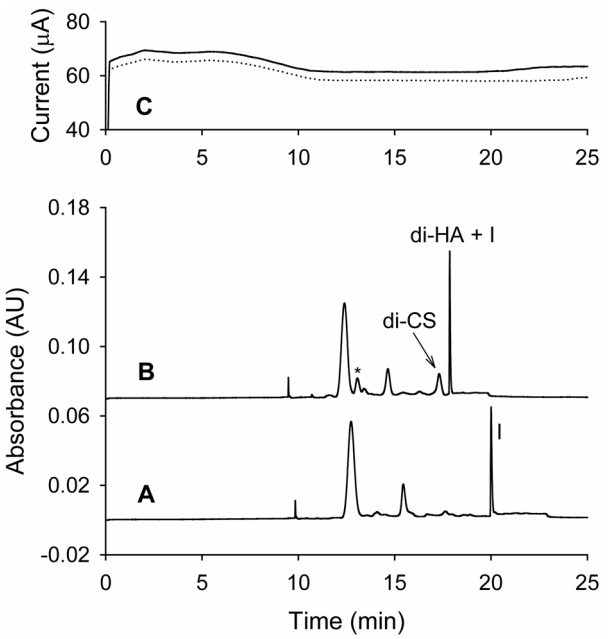


Figure 7. Electropherograms obtained for analysis of the serum of a healthy person (A) without enzymatic depolymerization but otherwise the same sample pretreatment and (B) after chondroitinase ABC depolymerization as described in Section 2.3. di-HA content was estimated to be 0.46 μg/mL. Current data are presented in panel C (dotted and solid lines for blank and depolymerized serum, respectively). Peak denoted with the letter I refers to a blank peak that interferes with the detection of di-HA. Experimental conditions as in Section 2.2.

sample with the standards of di-HA and di-CS followed by reanalysis. Furthermore, based on the work of Karamanos and Hjerpe [26], the peak marked with an asterisk in Fig. 7B could represent a sulfated disaccharide of CS. The identity of all other peaks is unknown. Using the calibrations discussed above that are based on corrected peak areas, the amount of di-HA in this serum was estimated as being 3.03 ± 0.16 μg/mL (average of three experiments). Similar results were obtained with other samples. Although a more accurate value could possibly be obtained *via* calibration with standard additions of di-HA to the prepared serum sample prior to reanalysis, this approach was not investigated as the di-HA levels found are more than one order of magnitude higher than expected. According to the protein binding assays used routinely in clinical laboratories [17], serum HA levels of normal subjects should be lower than about 100 ng/mL. Thus, not surprisingly, the di-HA peaks determined for the depolymerized serum samples were found to contain other tightly stacked compounds that comigrated with di-HA (denoted with letter I in Fig. 7). This could unambiguously be proven by analysis of the serum without chondroitinase ABC depolymerization, *i.e.*, during sample preparation, the Tris-HCl buffer was added without the

Table 2. Regression data, linear ranges, and RSDs[a)]

Analyte	Regression equation[b)]	Linear range, μg/mL	Correlation coefficients	RSD,%[c)]	
				Corr. area[d)]	Detection time
di-HA	$y = 957.2 + 226.3x$ $y = 41.51 + 562.8x$	1.0–400 0.1–1.0	0.9996 0.9978	2.13	1.24
di-CS	$y = 515.4 + 183.3x$ $y = 5.959 + 134.0x$	1.0–400 0.2–1.0	0.9994 0.9934	1.57	1.13

a) Experimental conditions as stated in Section 2.2 using samples composed of the two disaccharides in 25 mM Tris-HCl, pH 7.5.
b) Regression analysis was executed with mean values from three determinations. Concentrations and corrected peak areas were used as *x* and *y* variables.
c) $n = 9$ using a sample with 10 μg/mL of each disaccharide.
d) Peak area/detection time.

enzyme. A blank electropherogram is presented in Fig. 7A. The interfering blank peak (peak I in Fig. 7A) was found to be smaller compared to that detected after enzymatic depolymerization, thus permitting the di-HA content to be estimated *via* blank subtraction (subtraction of area of peak I of Fig. 7A from area of peak "di-HA + I" of Fig. 7B). For the example depicted in Fig. 7, the di-HA concentration was thereby determined to be 0.46 μg/mL. Using the Corgenix protein binding assay, the same serum revealed an HA level of 21 ng/mL. More work is required to provide insight into the difference of these results.

With samples prepared from different sera, the detection times of di-HA containing peaks were found to vary from sample to sample (between about 17 and 23 min), between sample and its blank (Fig. 7A and B), and to be significantly different to those observed with the standards in the Tris-HCl buffer (compare data of Figs. 5A, 7B). Differences in the sample matrices are believed to be responsible for this variation. For the analysis of samples containing various amounts of di-HA in the Tris-HCl buffer, the detection time intervals were found to be constant (Table 1; around 10 min, Fig. 5A). The recovery of di-HA was determined by addition of known amounts of the di-HA standard substance into the digested and reconstituted human serum sample. Forty microliters of 100 μg/mL di-HA in Tris-HCl (pH 7.5) and 40 μL of depolymerized and reconstituted human serum sample were mixed and analyzed. The mean ($n = 6$) recovery of di-HA and RSD were calculated to be 92.4 and 3.01%, respectively. The mean detection time and RSD were 14.66 min and 1.69%, respectively. The detection time is between those of the standards in Tris-HCl (Fig. 5A) and the patient sample (Fig. 7B), again indicating the impact of the sample matrix on the detection time.

Based upon the presented data, it is clear that di-HA in the prepared serum samples cannot be determined by CE without further simplification of the sample matrix. In our laboratory, efforts are currently being undertaken to investigate the nature of the endogenous compounds that comigrate with di-HA and to find effective means to eliminate the interfering blank peak. These results will be presented in an upcoming communication.

4 Concluding remarks

In conclusion, the investigated capillary electrophoretic method featuring a tetraborate buffer at pH 10, reversed polarity, and reversed EO is shown to provide good resolution of the nonsulfated disaccharides of HA and CS in model samples and serum digested with chondroitinase ABC. Electrokinetic injection from a sample dissolved in 25 mM Tris-HCl (pH 7.5) is demonstrated to provide a sharply stacked di-HA peak that can accurately and reliably be quantitated based upon corrected peak areas. With sample application at −10 kV for 40 s, the detection limit for di-HA is 0.075 μg/mL. Analysis of enzymatically depolymerized HA in human serum revealed a sharp peak containing di-HA and endogenous interferences such that the di-HA content could only be estimated *via* subtraction of the blank peak obtained with the undepolymerized serum. For direct analysis of di-HA, investigations leading to samples without comigrating interferences have to be undertaken.

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